J. Biochem. **139**, 557–561 (2006)
doi:10.1093/jb/mvj058

Experimental Basis for a Stable Plasmid, pLS30, to Shuttle between *Bacillus subtilis* Species by Conjugational Transfer

Nagayoshi Sakaya, Shinya Kaneko, Satoko Matsunaga and Mitsuhiro Itaya*
Mitsubishi Kagaku Institute of Life Sciences, 11 Minamiooya, Machida-shi, Tokyo 194-8511

Received December 4, 2005; accepted January 17, 2006

The use of *Bacillus subtilis* 168 as the initial host for molecular cloning and subsequent delivery of the engineered DNA to other *Bacillus* hosts appears attractive, and would lead to an efficient DNA manipulation system. However, methods of delivery to other *Bacillus* species are limited due to their inability to develop natural competence. An alternative, unexplored conjugational transfer method drew our attention and a *B. subtilis* native plasmid, pLS30, isolated from *B. subtilis* (*natto*) strain IAM1168 was characterized for this aim. The nucleotide sequence (6,610 bp) contained the *mob* gene and its recognition sequence, *oriT*, that features pLS30 as a mobile plasmid between *Bacillus* species on conjugational transfer. Plasmid pLS3001, a chimera with a pBR322-based plasmid prepared in *Escherichia coli* to confer an antibiotic resistance marker, showed apparent mobilizing activity in the pLS20-mediated conjugational transfer system recently established. The *rep* gene and associated *palT1*-like sequence common to all other pLS plasmids previously sequenced indicated that pLS30 is a typical rolling circle replicating (RCR) type plasmid. Due to the significant stability of pLS30 in IAM1168, application of a mobile plasmid would allow quick propagation to *Bacillus* species.

Key words: *Bacillus natto*, conjugational transfer, plasmid, rolling circle replication, transformation.

Abbreviations: CHEF, contour-clamped homologous electric field; AO, acridin orange.

Stable DNA cloning is one of the most basic tools in biology. In *Bacillus subtilis*, a Gram-positive endospore-forming bacterium, recombinant gene delivery and engineering via a plasmid have been successful due to the ability of *B. subtilis* 168 to develop natural competency (*1*, *2*). The astonishing high degree of this competent nature, in terms of structural fidelity and efficiency, allows the shuttling of DNAs between *B. subtilis* 168 strains. Hence, the use of *B. subtilis* 168 as the initial host for cloning (*3–5*) and the subsequent delivery of the engineered DNA to an other host of interest is novel and attractive (*6–8*). However, gene delivery to strains other than *B. subtilis* 168 that are unable to develop competency required an alternative efficient and general method.

We recently re-investigated the only reported conjugational system involving pLS20 for *B. subtilis* (*9*), and established a rapid and convenient DNA delivery protocol (*10*). As *B. subtilis* 168 possesses no endogenous plasmid, plasmid vectors conventionally used have been constructed based on non-native plasmids such as pUB110 and pC194, which were isolated from other Gram-positive bacteria, *Staphyrococci* and *Streptococci* (*2*). As these plasmids originated from apparent pathogens, scrutiny may be required for potential hazardous DNA sequences, which is currently less likely but still possible. Plasmids isolated from more closely related, generally-regarded-as-safe *B. subtilis* (*natto*) strains in Japan may have to replace them. Most *B. subtilis* (*natto*) small plasmids isolated including the pTA series (*2*) and pLS series (11) have been sequenced to date, and categorized by Bron and his colleagues (*2*). Based on the pTA series plasmid sequences as representatives, pLS series plasmids are classified as follows; pLS11 and 12 as the pTA1060 family; pLS13 as the pTA1040 family; pLS14 as the pTA1050 family; and pLS15, 17, 19, 24, and 26 as the pTA1015 family (*2*). pLS30 was not included in the classification. No extensive studies of these small pLS plasmids for genetic stability and mobility in conjugational transfer have been performed, although both features are critical for alternative cloning vehicles (*10*).

During our re-investigation of *B. subtilis* (*natto*) strains possessing pLS plasmids (*12*), we fortuitously discovered that pLS30 carried by IAM1168 (BEST323) exhibited unexpectedly high resistance to segregational loss in the presence of acridine orange (AO). IAM1168 carries two co-existing plasmids, small pLS30 and large pLS31, as shown in the lane 2 in Fig. 1 (*11*, *12*). On our screening to isolate cells that had lost only pLS30 by mini-scale plasmid isolation, expected segregants have never been obtained, whereas several strains that had lost pLS31 were found. Because pLS31, categorized as a pLS20 family plasmid (*11*, *12*), should be regarded as a stable replicon with a lower copy number according to its theta-type replication mode (*13*), more infrequent loss of pLS30 seemed curious to us. This apparent genetically stable nature of pLS30 prompted us to investigate its details with the motivation that pLS30 was the last small pLS plasmid remaining to be sequenced (*2*, *11*). The kinetics for the conjugational transfer activity of pLS30 were compared with those of non-native plasmid pUB110 (*9*),

*To whom correspondence should be addressed. Tel: +81-42-724-6254, Fax: +81-42-724-6254, E-mail: mita2001@sfc.keio.ac.jp

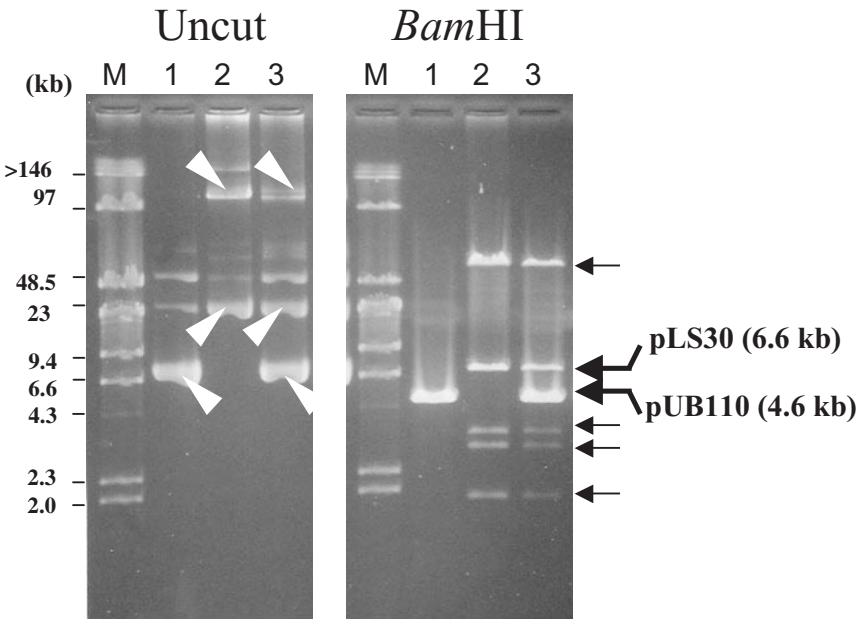

Fig. 1. ***B. subtilis* (*natto*) native plasmids.** Covalently closed circular (ccc) plasmids isolated by ultracentrifugation in the presence of ethidium bromide resolved by CHEF. Left: Lane 1, pUB110 (arrowhead) isolated from RM125; lane 2, pLS30 (bottom) and pLS31 (top) from IAM1168; and lane 3, plasmids from IAM1168 into which pUB110 was delivered by electroporation. Bands not indicated by arrowheads, possibly multimeric or relaxed forms of the three plasmids, are not specified. Right: Bold arrows indicate linear pUB110 and pLS30 obtained on *Bam*HI digestion. *Bam*HI fragments generated from pLS31 in lanes 2 and 3 are indicated by thin arrows on the right. Lambda and lambda/*Hin*dIII size markers are indicated on the left. The running conditions for CHEF were 1.0% agarose, 8 V/cm, 3 s pulse time and 4 h running time at 14°C.

bearing the *B. subtilis* native plasmid value for more general use in mind.

MATERIALS AND METHODS

Bacterial Strains and Plasmid—All the *B. subtilis* strains were derived from restriction-modification deficient strain RM125 (*arg leu*) (*14*) except for BEST2125 (*15*), which was derived from 1A1 (*trpC2*: from *Bacillus* Genetic Stock Center, Ohio). The pLS30 carrier IAM1168 (*11*, *12*) and *B. subtilis* 168 derivatives were grown in Luria-Bertani broth at 37°C. *Escherichia coli* JA221 (F^- *hsd*R *hsd*M *trp leu lacY recA1*) was routinely used as a host for molecular cloning. Preparation and transformation of competent *B. subtilis* were performed as previously described (*16*). Ampicillin (50 μg/ml) was added to the medium for JA221 selection. Tetracycline (10 μg/ml), chloramphenicol (5 μg/ml), kanamycin (5 μg/ml), and spectinomycin (50 μg/ml) were added for *B. subtilis* selection. pCISP410A (8,535 bp) was derived from pCISP310B (*4*) by insertion of a multiple restriction enzyme recognition sequence 5′-GATCCAGCTGGTACCA-CGCGT-3′ into the *Bam*HI site.

In Vitro DNA Manipulation—Type II restriction enzymes and T4 DNA ligase were obtained from Toyobo (Tokyo, Japan). The reaction of these enzymes and handling of the plasmid in *E. coli* were performed according to the manufacture's manual and the established method (*17*), respectively. Isolation of a plasmid from a *B. subtilis* culture was performed according to the SDS-alkali protocol developed for *E. coli*, modified by only increasing the lysozyme amount to 10 μg/ml. This mini-scale plasmid isolation protocol was also used to detect a plasmid with no antibiotic marker.

Plasmid Loss with Acridine Orange—IAM1168 cultivated in LB medium containing AO at 5 μg/ml at 37°C was spread on a fresh LB plate after appropriate dilution to form colonies. Strains that had lost pLS30 or pLS31, or both were screened by observation of plasmids. In one screening, three strains for pLS31 loss, BEST40158 as a representative, and none for pLS30 loss were found among 13 colonies.

Isolation and Characterization of pLS30—pLS30 and other recombinant plasmids were purified on a CsCl density gradient formed through ultracentrifugation in the presence of ethidium bromide (*17*). A large amount of pLS30 was prepared from strain BEST40158, which lacks pLS31. Based on a restriction enzyme site map constructed for the purified pLS30 (data not shown), five *Hin*dIII fragments of pLS30 were subcloned into the *Hin*dIII site of the pBR322 plasmid in *E. coli* JA221 by the standard cloning method (*17*). DNA sequencing with synthetic primers was conducted with an ABI3100 sequencer (Applied Biosystems, CA, USA) and Big Dye terminators. The PCR primers were prepared by DATE Concept Co. (Sapporo, Japan).

*Kinetics for Plasmid Transfer under pLS20*cat*-Mediated Conjugational Transfer Assay Conditions*—pLS20-mediated conjugational transfer was carried out according to a protocol (*10*) involving BEST2125 (*trpC2 proB*::pBRTc) as a recipient. The recipient can be selected with tetracycline (Tc) due to the resistance gene (*tetL*) inserted in the *proB* gene (*15*). BEST40401,which is resistant to Cm due to the presence of the pLS20*cat* replicon (*10*), was transformed with pUB110 (Km^R) or pLS3001 (Spc^R) to yield a donor strain, BEST40402 or BEST40411. *B. subtilis* cultures grown at 37°C for 15–17 hours in LB medium (BEST2125) or LB medium containing Cm and Km (BEST40402), or Spc (BEST40411) were diluted 1:20 in 20 ml pre-warmed LB in 150-ml flasks and then shaken at 120 rpm at 37°C. Aliquots (0.1 ml) of donor and recipient cells were mixed together at the indicated times and kept for 15 min at 37°C. An appropriate volume of a mating mixture was spread on a LB plate supplemented with Km and Tc for a pUB110 transcipient or with Spc and Tc for a pLS3001 transcipient. Colonies formed after incubation at 37°C for 24 h were scored.

RESULTS AND DISCUSSION

Characterization of pLS30—The complete 6,610 bp sequence of pLS30 purified from BEST40158 was determined as described under "MATERIALS AND METHODS," and deposited in DDBJ. Unveiled were five open reading frames (ORFs), as shown in Fig. 2, homologues of which were all found among the pLS plasmids characterized to date (*1*, *2*). The *rep* gene should be essential for vegetative rolling circle replication (RCR). The presence of a putative double stranded origin (DSO) and a *palT1*-like single stranded origin (SSO) normally associated with RCR plasmids also supports this replication mode for pLS30 (*1*, *2*, *18*, *19*). Putative hsp, present in some of RCR plasmids, belongs to the ubiquitous family of small proteins whose members have so far been associated with stress response (*19*). The functions of the putative transmembrane protein and DNA binding protein remain obscure. The largest protein encoded by the *mob30* gene, 482 amino acid residues of 56.6 kDa, exhibits certain similarity to proteins in another *mob* gene family summarized in Fig. 6 in Ref. *2*. *mob30* is identical to *mob15* found in pLS15, 17, 19, 24, and 26, and 97% similarity to *mob60* was found for pLS11 and 12 (*2*). Only a few plasmids possessing the *mob* gene such as pUB110 had been experimentally proven by Koehler and Thorne (*9*). No quantitative evaluation of conjugational transfer activity had been described for any pLS plamid. We established a protocol for pLS20-mediated conjugational transfer assaying in liquid culture by which quantitative comparison is possible (*10*).

Shuttling plasmid pLS3001—To measure quantitative conjugational transfer activity, an appropriate selective marker should be conferred on pLS30. To this end and for its versatile use as a shuttling plasmid for *E. coli*, a pBR322-derived *E. coli* plasmid, pCISP410A (described under "MATERIALS AND METHODS", and shown in Fig. 2) was inserted in the unique *Kpn*I site of pLS30. The ligated product with the *Kpn*I fragment of pCISP410A was used to transform *B. subtilis*, and colonies resistant to spectinomycin and chloramphenicol yielded a chimera plasmid designated as pLS3001 (15,145 bp), as shown in Fig. 2. *B. subtilis* strain RM125 carrying pLS3001 appeared at 100% in the presence and 92 ± 6% in the absence of spectinomycin after 7 generations. Since genetic stability or maintenance of a delivery plasmid is critical for conjugation, the apparent stability in the presence of spectinomycin was suitable for the quantitative conjugational transfer experiment, albeit the reduced stability without antibiotic selection promoted us to examine the original pLS30 carrier strain, IAM1168, as described below.

Altered Stability of pLS30 in a Different Genetic Background—The stability of pLS30 in cognate host IAM1168 was measured by screening for presence of plasmids because no markers were to be monitored. On screening by mini-scale plasmid isolation, 200 randomly selected colonies formed after 7 generations in LB medium at 37°C all possessed both pLS30 and pLS31. The lack of spontaneous segregants, consistent with our observation of little segregation when exposed to AO, was in sharp contrast to the reduced stability (92 ± 6%) of pLS3001 in *B. subtilis* 168 in the absence of spectinomycin. To examine the possible difference in genetic background between IAM1168 and *B. subtilis* 168, transfer of pLS3001 to IAM1168 by electroporation was conducted. However, all attempts to deliver pLS3001 into IAM1168 were unsuccessful in contrast to the efficient delivery of pUB110, as

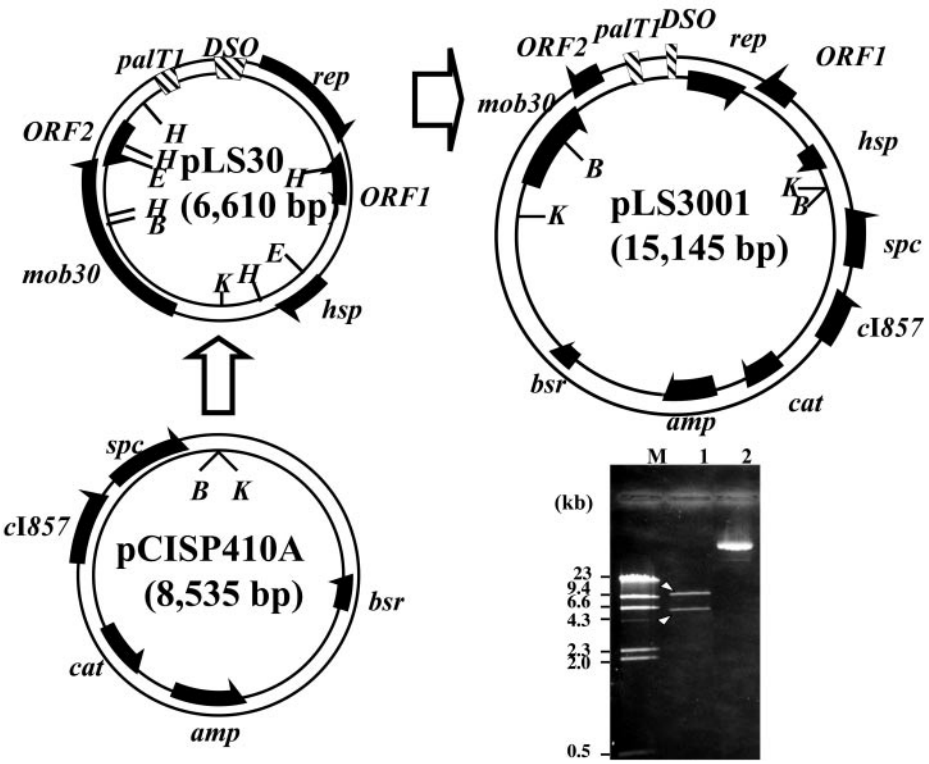

Fig. 2. **Structures of plasmid pLS30 and its derivative pLS3001.** Location of the primary and secondary replication origins, DSO and *palT1*, according to reference 2. Restriction enzyme sites *Bam*HI, *Eco*RI, *Hin*dIII and *Kpn*I are designated as B, E, H, and K. ORF1 and ORF2 encode a putative transmenbrane protein and a putative DNA binding protein, as suggested in reference 2. pLS3001 prepared from *B. subtilis* is able to replicate in *E. coli*. Genes *amp*, *bsr*, *cat*, *c*I 857 are blasticidin resistance (*4*), beta lactamase (*17*), chroramphenicol acetyl transferase (*4*), and lambda *c*I (*4*, *5*) genes. The running conditions for CHEF that included pLS3001 digested (lane 1) and undigested (lane 2) by *Bam*HI were 1.0% agarose, 6 V/cm, 3 s pulse time and 3 h running time at 14°C.

indicated in lane 3 in Fig. 1. Because IAM1168 does not develop genetic competency, no other attempt to replace pLS30 by pLS3001 was made. This observation implied an incompatibility nature for pLS30 and/or an internal mechanism specific to strain IAM1168 such as a restriction-modification system, albeit size-dependent efficiency in electroporation cannot be ruled out. This adds certain weight to the evidence that small *Bacillus* plasmids may not be cryptic but may provide an adaptive advantage for a host in its natural environment (*18*, *19*). The pLS30 stability may be caused by pLS31-mediated conjugation, which minimizes the subpopulation of pLS30-free cells. This idea stems from that a plasmid possessing a *mob* gene homologue together with a co-existing pLS20 family plasmid in *B. subtilis* (*natto*) (*11*, *12*) can contribute to horizontal plasmid DNA pools of a *Bacillus* bacterial population. Genes carried by such plasmids may facilitate molecular adaptation of the host to certain environmental conditions, as discussed in some references (*1*, *2*, *18*, *19*). Concerning this, it should be mentioned that, during our pLS30 study, we found a sequence, pBS608, isolated from a *B. subtilis* strain by Chen and Gao (DDBJ accession number AY836798, deposited on Dec. 20, 2004). pBS608 (6,611 bp) is nearly identical with pLS30, there being three nucleotide differences: G to C at 1,098, C to T at 5,422, and insertion of A between 2665 and 2666 of the pBS608 sequence that does not affect the ORF and functional sequences. The extreme similarity of pLS30 with pBS608 may indicate that they were derived from a common ancestor, albeit no information concerning the *B. subtilis* host of pBS608 is available.

Conjugational Transfer Activity of pLS3001—Donors harbouring pLS20*cat* (Cm^R) and pUB110 (Km^R) (BEST40402) or pLS20*cat* (Cm^R) and pLS3001 (Spc^R) (BEST40411) grown in LB medium supplemented with appropriate antibiotics were mixed with recipient BEST2125 (Tc^R), and then transcipients were selected as described under "MATERIALS AND METHODS." The numbers of transcipient cells selected with appropriate antibiotic resistance markers were plotted against time, as shown in Fig. 3. The transfer of pUB110 or pLS3001 to BEST2125 was confirmed by plasmid extraction of at most 6 randomly selected transcipients. The pUB110 transcipient appeared at an early growth stage, *i.e.*, faster than pLS20*cat*, and reached a maximum at 2 h cultivation, and then gradually decreased within 4 h, exhibiting a similar growth stage–specific profile to that of pLS20*cat* self transfer (*10*). Likely, the time-dependent appearance of pLS3001 trancipients similar to the conjugational kinetics of pUB110, as shown in Fig. 3, strongly indicates that Mob30-*oriT30* of pLS30 is functional. The significantly reduced frequency of pLS3001, by approximately three orders of magnitude, compared with pUB110 (4.55 kb) remains to be explained. A size increase of pLS3001 (15.1 kb) is less likely because of the higher rate for 65 kb pLS20*cat*. Rather, different levels of functional activity of the Mob protein *in vivo* might be due to the reduction. The similarity of Mob_{pUB110} to Mob30 of 66.1%, as indicated in Fig. 6 in Ref. *2*, is consistent with the slightly different Mob recognition sequence, TTTGGTGTAGTGCGT/TACACCAAA, in *oriT30* from that, TAAAGTATAGTGTGT/TATACTTTA, in $oriT_{pUB110}$. The underlined sequences form the stem structure to be recognized by Mob protein (*2*). The finding that the conjugational activity for the internal Mob30-*ori*T30 combination in the closely related pLS20 conjugational system is dramatically reduced compared with the external Mob_{pUB110}-$oriT_{pUB110}$ one is likely paradoxical and needs further experimental study.

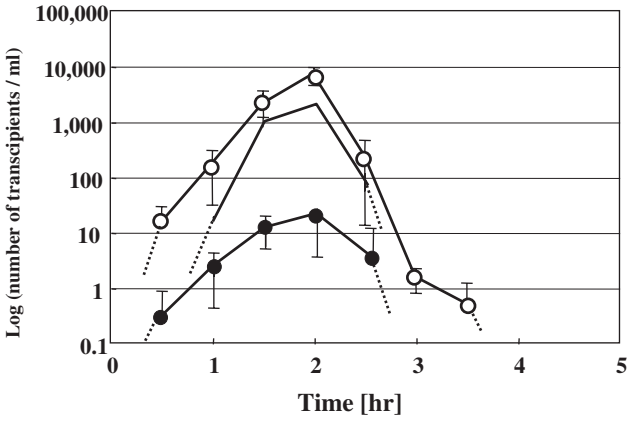

Fig. 3. **Conjugational property of pLS3001 by pLS20-mediated conjugational transfer kinetics in liquid media.** The numbers of transcipients of pUB110 at the indicated times for BEST2125 (open circles) as the recipient. The time-dependent numbers of pLS3001 with the standard protocol are shown by (solid circles). A mating experiment with an equal volume of a growing culture was performed every 30 min. The time-dependent numbers of pLS20*cat* transcipients with the same protocol are shown by a simple solid line, as reported in reference 10. No transcipients at other times are indicated by dotted lines.

Application of this mobile plasmid will allow quick delivery of DNA clones assembled in a *B. subtilis* genome vector to other *Bacillus* species (*3*, *20*, *21*). The transfer of several genes, including antibiotic resistance genes, via the pLS30 vector indicates our goal is being achieved (data not shown).

Nucleotide sequence data of pLS30 are available in the DDBJ/EMBL/GenBank databases under accession number AB243053. This work was supported by a research grant (No. 16380066) from the Ministry of Education, Culture, Sports, Science and Technology.

REFERENCES

1. Bron, S. (1990) Plasmids in *Molecular Biologaical Methods for Bacillus* (Harwood, C. and Cutting, S., eds.) pp. 75–138, Johan Wiley & Sons, New York
2. Meijer, W.J.J., Wisman, G.B., Terpstra, P., Thorsted, P.B., Thomas, C.M., Holsappel, C.M., Venema, G., and Bron, S. (1998) Rolling-circle plasmids from *Bacillus subtilis*: complete nucleotide sequences and analyses of genes of pTA1015, pTA1040, pTA1050, and pTA1060, and comparisons with related plasmids from Gram-positive bacteria. *FEMS Microbiol. Rev*. **21**, 337–368
3. Kaneko, S., Akioka, M., Tsuge, K., and Itaya, M. (2005) DNA shuttling between plasmid vectors and a genome vector: Systematic conversion and preservation of DNA libraries using the *Bacillus subtilis* genome (BGM) vector. *J. Mol. Biol.* **349**, 1036–1044
4. Itaya, M., Fujita, K., Koizumi, M., Ikeuchi, M., and Tsuge, K. (2003) Stable Positional Cloning of Long Continuous DNA in the *Bacillus subtilis* genome vector. *J. Biochem.* **134**, 513–519
5. Itaya, M., Tsuge, K., Koizumi, M., and Fujita, K. (2005) Combining two genomes in one cell: Stable cloning of the

cyanobacterium PCC6803 genome in the *Bacillus subtilis* 168 genome. *Proc. Natl. Acad. Sci. USA* **102**, 15971–15976
6. Tsuge, K. and Itaya, M. (2001) Recombinational transfer of 100-kb genomic DNA to plasmid in *Bacillus subtilis* 168. *J. Bacteriol.* **183**, 5453–5456
7. Tomita, S., Tsuge, K., Kikuchi, Y., and Itaya, M. (2004) Targeted isolation of a designated region of the *Bacillus subtilis* 168 genome by Recombinational transfer. *Appl. Environ. Microbiol*. **70**, 2508–2513
8. Tsuge, K., Matsui, K., and Itaya, M. (2003) One step assembly of multiple DNA fragments with designed order and orientation in *Bacillus subtilis* plasmid. *Nucleic Acids Res.* **31**, e-133
9. Koehler, T.M. and Thorne C.B. (1987) *Bacillus subtilis* (*natto*) plasmid pLS20 mediates interspecies plasmid transfer. *J. Bacteriol.* **169**, 5271–5278
10. Itaya, M., Sakaya, N., Matsunaga, S., Fujita, K., and Kaneko, S. (2005), Conjugational transfer kinetics for *Bacillus subtilis* in liquid culture. *Biosci. Biotechnol. Biochem*. in press
11. Tanaka, T. and Koshikawa, T. (1977) Isolation and characterization of four types of plasmids from *Bacillus subtilis* (*natto*). *J. Bacteriol.* **131**, 699–701
12. Qiu-Dongru, Fujita, K., Sakuma, Y., Tanaka, T., Ohashi, Y., Ohshima, H., Tomita, M., and Itaya, M. (2004) Comparative analysis of the structure of complete physical maps of four *Bacillus subtilis* (natto) genome. *Appl. Environ. Microbiol.* **70**, 6247–6256
13. Meijer, W.J., Boer, A.J., Tongeren S., Venema, G., and Bron, S. (1995) Characterization of the replication region of the *Bacillus subtilis* plasmid pLS20: a novel type of replicon. *Nucleic Acids Res*. **23**, 3214–3223
14. Itaya, M. and Tanaka, T. (1997) Predicted and unsuspected alterations of the genome structure of genetically defined *Bacillus subtilis* 168 strains. *Biosci*. *Biotech*. *Biochem*. **61**, 56–64
15. Itaya, M. (1993) Integration of repeated sequences (pBR322) in the *Bacillus subtilis* 168 chromosome without affecting the genome structure. *Mol. Gen. Genet.* **241**, 287–297
16. Anagnostopoulos, C. and Spizizen, J. (1961) Requirements for transformation in *Bacillus subtilis*. *J. Bacteriol.* **81**, 741–746
17. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
18. Meijer, W.J., Jong, A., Bea, G., Wisman, A., Tjalsma, H., Venema, G., Bron, S., and Dijl, J.M. (1995) The endogenous *Bacillus subtilis* (*natto*) plasmids pTA1015 and pTA1040 contain signal peptidase-encoding genes: identification of a new structural module on cryptic plasmids. *Mol. Microbiol*. **17**, 621–631
19. Thorsted, P.B., Thomas, C.M., Polueltova, E.U., and Prozorov, A.A. (1999) Complete sequence of *Bacillus subtilis* plasid p1414 and comparison with seven other plasmid types found in Russian soil isolated of *Bacillus subtilis*. *Plasmid* **41**, 274–281
20. Itaya, M., Nagata, T., Shiroishi, T., Fujita, K., and Tsuge, K. (2000) Efficient cloning and engineering of giant DNAs in a novel *Bacillus subtilis* genome vector. *J. Biochem.* **128**, 869–875
21. Kaneko, S., Tsuge, K., Takeuchi, T., and Itaya, M. (2003) Conversion of submegasized DNA to desired structures using a novel *Bacillus subtilis* genome vector. *Nucleic Acids Res.* **31**, e112